

## SELF-REGULATION OF GROWTH IN THREE DIMENSIONS\*

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It is well known that growth of tumor cells in flat tissue culture is unrestricted, provided that medium is replenished frequently and sufficient open space is made available. This persistent growth continues only in two dimensions and is not self-regulating.<sup>1</sup>

It has not been appreciated that growth in three dimensions is self-regulating. We show that cells when removed from a plane surface and forced to grow in three dimensions in spheroidal or ellipsoidal population, will not expand beyond a critical diameter and cell number, regardless of how often new medium is provided or how much open space is made available.

Sutherland et al. (1), Dalen and Burki (2), and Shinohara and Okada (3) have studied multi-cell spheroids in suspension and semi-solid culture as an analog to nodular carcinomas in vivo. They grew multiple spheroids in each flask. In repeating these experiments, we found spheroid diameter to be inversely proportional to the number of spheroids in a flask (Fig. 1). However, a single spheroid, isolated from the crowd and repeatedly transferred to new medium, achieved a larger diameter. The isolated spheroid eventually reached a maximum mean diameter beyond which no further expansion was possible no matter how often the medium was replenished. This was called the "dormant" diameter.

### *Methods*

B-16 mouse melanoma, V-79 chinese hamster lung, and L-5178Y murine leukemia cells were plated in soft agar (5). 6 ml of Fischer's medium at 44°C, containing 0.2% Noble agar, 10% horse serum, and antibiotics, were added to Falcon flasks (25 mm<sup>2</sup>) which were previously gassed with 5% CO<sub>2</sub>. The flasks were removed to room temperature and 4 ml of Fischer's medium with 15% horse serum containing 100 cells per milliliter were added to each flask to give a final concentration of 0.12% agar. The cells were dispersed by gentle mixing. The agar was allowed to gel at 4°C for 5 min and then the flasks were incubated at 37°C. After 6-7 days, spheroidal colonies of 0.1 mm were visible. Each spheroid was transferred with a wide bore

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<sup>1</sup> The third dimension, thickness, quickly reaches a steady-state dependent upon the particular cell strain. However, even the most malignant cells in culture rarely pile up more than 5-6 layers deep. (Unless medium is changed continuously by perfusion [4].)

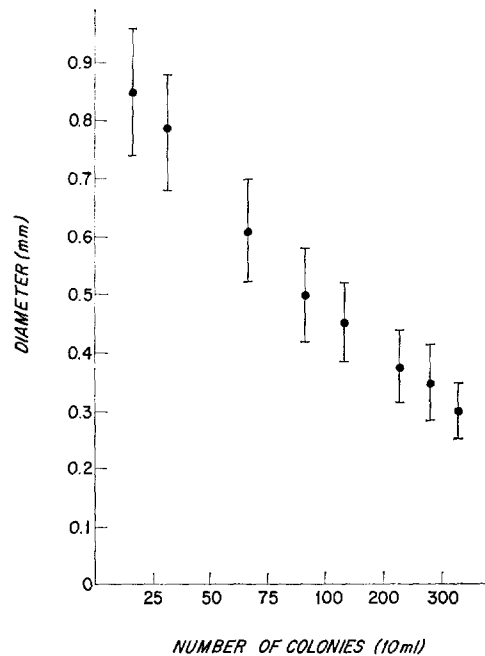


FIG. 1. Mean diameter and standard deviation of spheroidal colonies of L-5178Y murine leukemic cells in 10 ml soft agar. Diameter is inversely proportional to the number of spheroids.

pipette to a new flask containing 10 ml of fresh nutrient medium in 0.12% agar previously gelled. Transfers to a flask containing fresh agar were repeated every 2-3 days. This short interval prevented any pH change. The flasks were always incubated in the upright position, and the spheroid suspended about equal distance between the bottom of the flask and the surface of medium. The diameter of each spheroid was measured every 2-3 days by projecting its image on a white table top at 20 $\times$  magnification. 10 thousand transfers of spheroids to new medium were made over a period of 1 yr.

#### *Experiments and Results*

All spheroids first enlarged exponentially for a few days, and then continued on a linear growth curve for 5-23 wk before reaching a critical diameter beyond which there was no further expansion, i.e., the dormant phase. For L-5178Y cells, the mean diameter of the dormant phase was  $3.8 \text{ mm} \pm 0.5 \text{ mm}$  at approximately 24 days (Fig. 2 *a*); for V-79 cells, the dormant diameter was  $4.0 \text{ mm} \pm 0.8 \text{ mm}$ , reached at 175 days (Fig. 2 *b*), and for B-16 melanoma cells, it was  $2.4 \text{ mm} \pm 0.4 \text{ mm}$  reached at approximately 100 days (Fig. 2 *c*).

Spheroids were removed periodically from two groups and dispersed (trypsinisation for V-79, simple shaking for L-5178Y) in 0.5 ml Ringers solution containing a crystal of nigrosin for viable and total cell counts. The maximum viable cell count for L-5178Y was  $3 \times 10^5$  cells at 20 days (Fig. 3 *a*); and for V-79,  $2 \times 10^5$  cells at 45 days (Fig. 3 *b*).

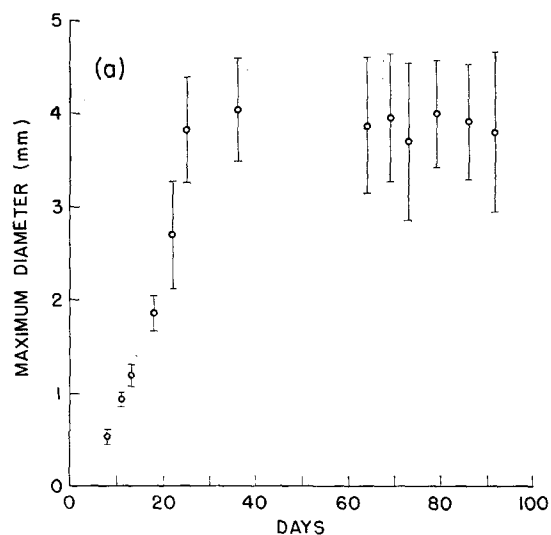


FIG. 2 *a*. Mean diameter and standard deviation of 50 isolated spheroids of L-5178Y cells. Each spheroid was maintained in 10 ml of soft agar and transferred every 2-3 days to a new flask.

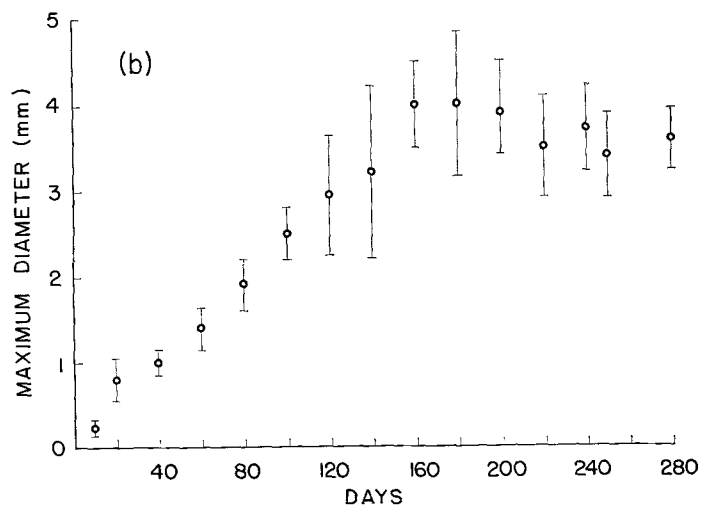


FIG. 2 *b*. Mean diameter and standard deviation of 70 isolated spheroids of V-79 cells, treated similarly to the L-5178Y cells. Very old spheroids occasionally shattered and were discarded. Therefore, mean diameter after 200 days represented approximately 30 colonies.

Throughout the dormant phase, a portion of the population in each spheroid remained viable as demonstrated by the following: (*a*) Dormant spheroids reduced pH of the medium if they were not transferred to a new flask, i.e., a dormant L-5178Y spheroid could reduce the pH of 10 ml of medium from 7.35 to

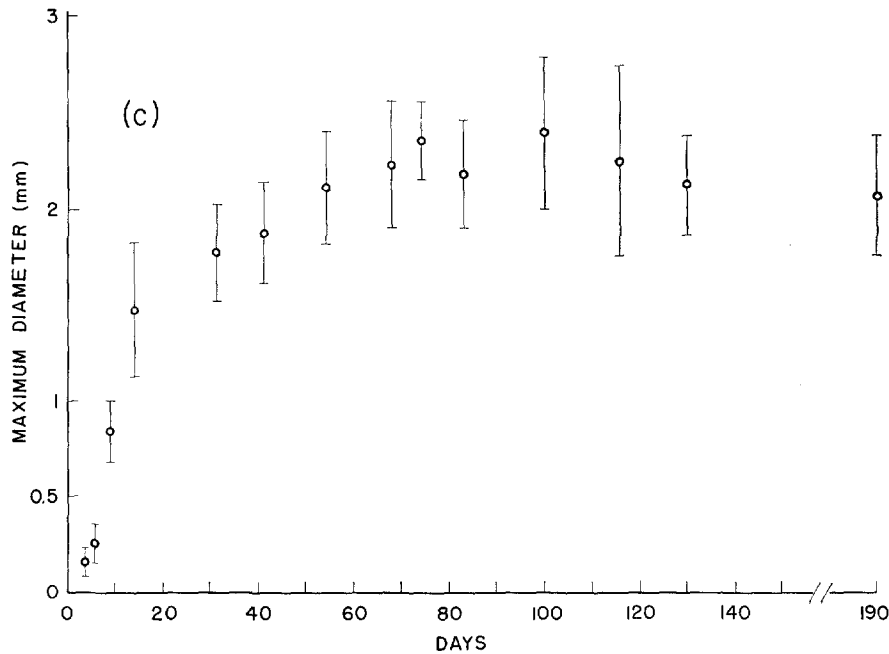


FIG. 2 c. Mean diameter and standard deviation of 32 isolated spheroids of B-16 melanoma.

6.85 in 8 days. (b) When a dormant spheroid was dispersed into single cells by shaking in fresh medium, pH was reduced by 0.4 U over 1 day. (c) New spheroids or monolayer cultures could be produced by cells shed from dormant spheroids. (d) Histologic sections of dormant spheroids always showed a central necrotic zone, a middle viable zone, and an outer zone of cells with mitotic figures.

Spheroids at various ages were transferred to fresh agar containing [ $^3\text{H}$ ]thymidine, 1  $\mu\text{Ci}/\text{ml}$ . After 4 h, they were fixed in 2.5% glutaraldehyde-2% paraformaldehyde in 0.1 M cacodylate buffer (pH, 7.4) for 4 h, washed overnight in cacodylate buffer, post fixed in 1% osmium tetroxide, dehydrated, and imbedded in Epon. 1  $\mu\text{m}$  thick Epon sections were developed as autoradiographs according to standard techniques (6) and stained with 1% toluidine blue. In spheroids under 1.0 mm diameter, almost all cells on the surface and five layers deep were labeled with [ $^3\text{H}$ ]thymidine (Fig. 4 a). As these spheroids approached the dormant size, the number of labeled cells diminished until they occupied only a single layer on the outermost rim of the spheroid (Fig. 4 b). The ratio of the volume of labeled cells to the total volume of the spheroid, decreased steadily with age, until the dormant phase was reached.

Dormant spheroids were pulsed with [ $^3\text{H}$ ]thymidine for 7–24 h and then cultured for 7 days in fresh medium. Labeled cells were found in the middle of the spheroids and labeled nuclei in the necrotic zone, but there was no label in the

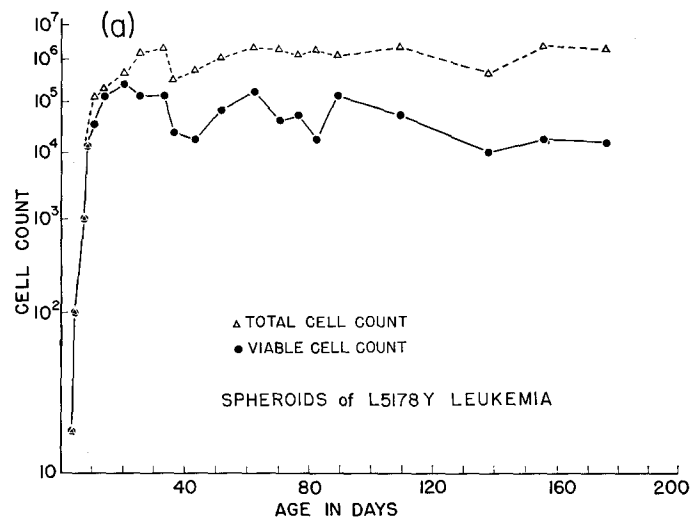


FIG. 3 *a*. The total number of cells and the number of viable cells in L-5178Y spheroids reach a steady-state at approximately the time that diameter of the spheroid enters the dormant phase.

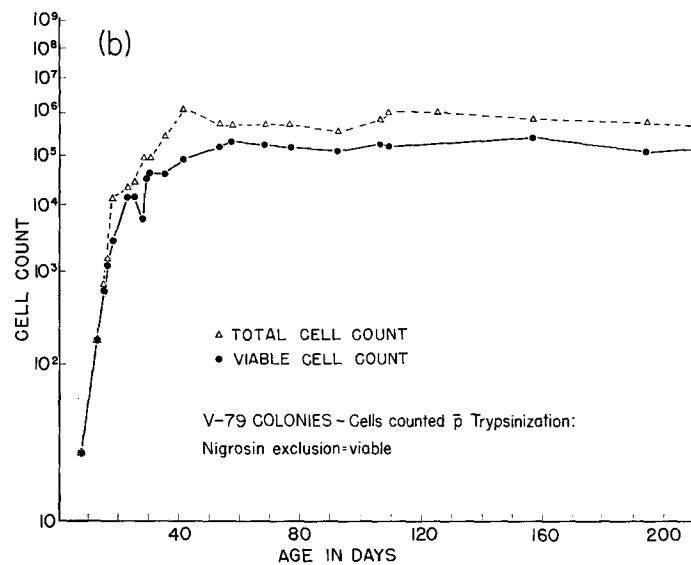


FIG. 3 *b*. The number of total cells and the number of viable cells in V-79 spheroids reach a steady-state 130 days before the spheroid stops expanding. Thus, a relatively constant number of viable cells is distributed over a gradually enlarging necrotic mass. This implies that lysis of necrotic debris does not reach equilibrium with production of new cells until approximately 175 days.

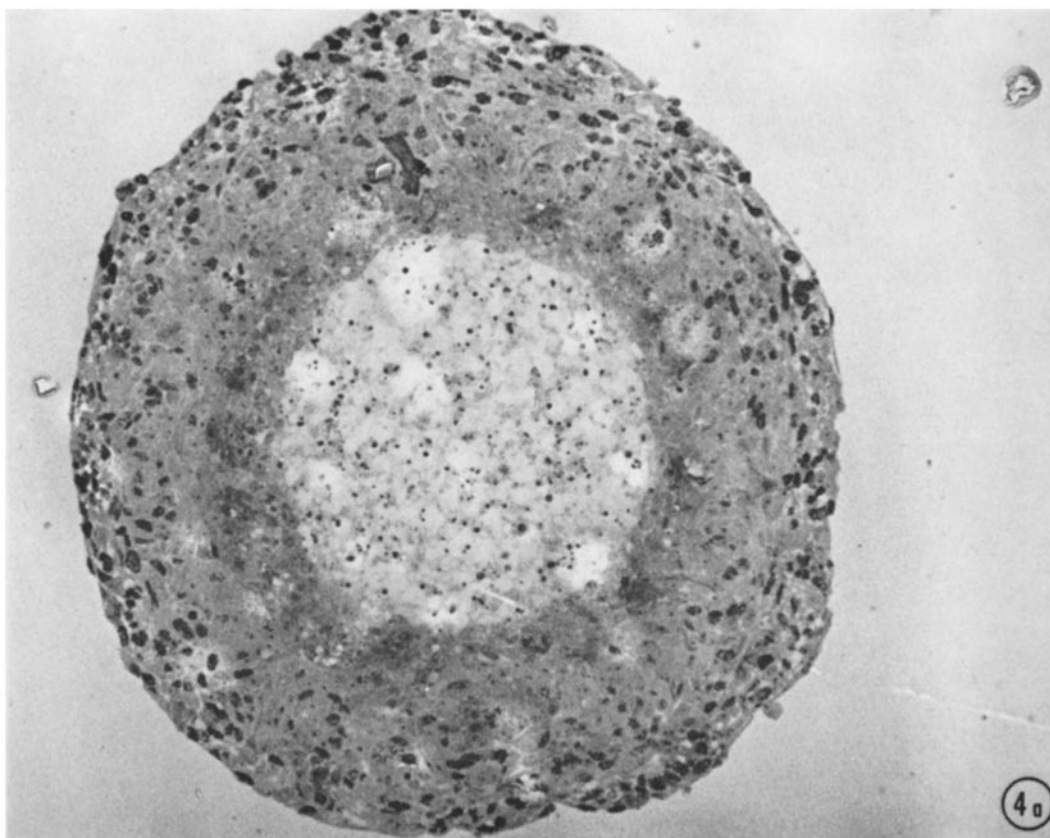


FIG. 4 *a*. Cross section of V-79 spheroid, 1.0 mm diameter and 20 days old. Five to seven outer layers of cells are labelled with [ $^3\text{H}$ ]thymidine.

$$\frac{\text{Volume of labeled cells}}{\text{Total Volume}} = 0.60.$$

outer zone. This indicated that in the dormant state, some cells newly generated on the surface, gradually migrate toward the center of the spheroid.

When dormant spheroids were exposed continuously for 7 days to high concentrations of [ $^3\text{H}$ ]thymidine (2  $\mu\text{Ci/ml}$ ), there was rapid regression in the size of the spheroid along a steep, linear slope.<sup>2</sup> This indicated that inhibition of mitosis in cells at the periphery of the spheroid prevented replacement of cells undergoing continual lysis in the necrotic center.

<sup>2</sup> Abraham, E., and J. Folkman. Unpublished observation.

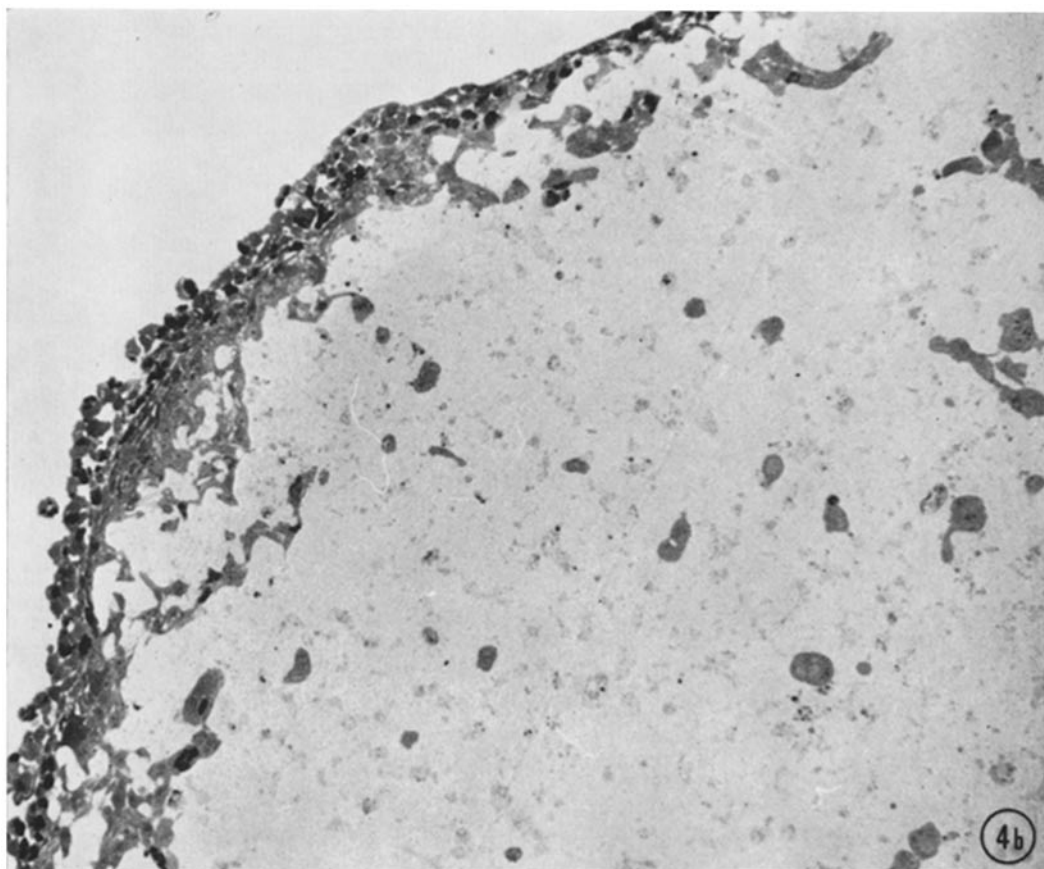


FIG. 4 *b*. Cross section of V-79 spheroid, 3.2 mm diameter and 150 days old. Only one or two outer cell layers are labeled with [ $^3\text{H}$ ]thymidine.

$$\frac{\text{Volume of labeled cells}}{\text{Total Volume}} = 0.14.$$

#### DISCUSSION

Therefore, spheroidal growth of cells in the presence of unlimited fresh medium and space appears to proceed in three phases: (*a*) a brief phase of exponential growth before the onset of central necrosis; (*b*) a second phase of linear growth beginning with the appearance of central necrosis. The first cells to die are greater than 150–200  $\mu\text{m}$  from the surface of the spheroid. This is most probably due to limitation of oxygen diffusion as previously described for packed cells (7); (*c*) a third or dormant phase which begins when the spheroid ceases to expand further. Throughout this phase, a small number of mitosing cells on the

surface maintain equilibrium with cells being lost from necrosis and shedding. The proliferating cells on the surface are essential to maintain the fixed volume of the sphere; if they are killed by toxic levels of [ $^3\text{H}$ ]thymidine, the spheroid rapidly shrinks. We propose that the mechanism of the dormant phase is based upon the diminishing ratio of surface area to volume which accompanies progressive growth of the spheroid.

At the moment dormancy begins, the volume of cells has reached a point where their aggregate surface area is insufficient to allow adequate escape of catabolites (or inhibitors) and absorption of nutrients. These data do not decide which phenomenon is primarily responsible for the arrest of growth, inhibitor accumulation (8, 9), or nutrient deficiency. But, they do make a strong case that spheroidal growth is self-regulatory.

By contrast, when cells grow in two dimensions in a plane, the ratio of surface area to volume remains constant in the face of unlimited fresh medium and space. There is always sufficient surface area so that within the cell population, catabolites do not accumulate, and nutrients are not depleted. Thus, growth in two dimensions is not self-regulating.

*Implication for Solid Tumors In Vivo.*—Many solid tumors in animals can be found—before they are vascularized—to be living as tiny spheroids or ellipsoids of a few millimeters diameter, dependent on simple diffusion for absorption of nutrients and release of catabolites (10).

We have shown (11, 12)<sup>3</sup> that when solid tumors are prevented from vascularization, they enter a dormant phase and do not exceed 1–2 mm in diameter. Their appearance is similar to dormant spheroids in culture, including the outer proliferating zone and the central necrotic zone. Only if new capillaries *pene-trate* the spheroid in vivo can the spheroid escape from the dormant phase and again begin rapid exponential growth.

Because most solid tumors in animals and man begin life as three-dimensional spheroids or ellipsoids, it is conceivable that angiogenesis is essential for a tumor to succeed and that “antiangiogenesis” (10), by whatever means, might force a tumor to remain in the dormant phase, at a tiny diameter of a few millimeters and a population fewer than  $10^6$  cells.

#### SUMMARY

Multi-cell spheroids were grown in soft agar. When each spheroid was cultured in a large volume of medium, frequently renewed, all spheroids eventually reached a dormant phase at a diameter of approximately 3–4 mm and a population of approximately  $10^6$  cells. In the dormant spheroid, newly generated cells at the periphery balanced those lost by necrosis in the center. We propose that this dormant phase is due to a gradual reduction in the ratio of surface area to volume: a size is achieved beyond which there is insufficient surface area for the

<sup>3</sup> Knighton, D., and J. Folkman. Unpublished observation.



spheroid to eliminate catabolites and absorb nutrients. Thus, in the face of unlimited space and of new medium, three-dimensional cell populations become self-regulating. This phenomenon contrasts with standard tissue culture in which cell populations, living on a flat plane in two dimensions, will not stop growing in the face of unlimited space and new medium because the ratio of surface area to volume remains constant.

These experiments provide a mechanism for our observations *in vivo*: before vascularization, solid tumors live by simple diffusion as three-dimensional spheroids or ellipsoids. They become dormant at a diameter of only a few millimeters; once vascularized, they are released from this dormant phase and begin exponential growth. Thus, tumor dormancy resulting from absence of angiogenesis *in vivo*, may operate by the same mechanism responsible for dormancy of spheroids *in vitro*.

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